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The *p53* gene is a potent determinant of chemosensitivity and radiosensitivity in gastric and colorectal cancers

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Abstract We previously reported that introduction of the wild-type *p53* gene into human cancer cells with deleted *p53* enhanced apoptosis induced by chemotherapy [Fujiwara et al. (1994) Cancer Res 54:2287]. This suggests that *p53* status could be a potent determinant of the therapeutic efficacy of DNA-damaging cancer therapy. We analyzed 24 patients with gastric or colorectal cancer for *p53* mutations and apoptotic changes in surgical specimens. Out of 11 patients with gastric cancer, 3 were treated with chemotherapeutic drugs before resection; 5 of 13 patients with colorectal cancer had 30 Gy radiation prior to surgery. *p53* mutations were detected in 4 cases of gastric cancer (36.4%) and in 6 cases of colorectal cancer (46.2%) by immunohistochemical staining. The preoperative DNA-damaging therapies increased the number of apoptotic cells in wild-type-*p53*-expressing tumors; tumors with mutant *p53*, however, significantly showed fewer apoptotic cells compared with those expressing wild-type *p53*. The *p53*-inducible WAF1/CIP1 protein was immunohistochemically observed in wild-type-*p53*-containing tumors, whereas mutant-*p53*-expressing tumors expressed no detectable WAF1/CIP1. Taken together, we conclude that *p53* mutations are associated with the poor response of chemotherapy and radiotherapy.

Key words *p53* · Chemoradiosensitivity · Apoptosis · Gastric cancer · Colorectal cancer

Abbreviations *TUNEL* terminal deoxynucleotidyltransferase-mediated biotin-dUTP nick-end labeling

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Introduction

Apoptosis (programmed cell death) was originally identified as an active process of self-destruction that occurs in organogenesis. Recent studies have demonstrated that a variety of DNA-damaging stimuli, such as ionizing radiation and chemotherapeutic drugs, initiate pathways leading to apoptosis in cancer cells (Kaufmann 1989). It is now accepted that apoptosis is a gene-directed process and associated with several second-messenger systems that regulate this process either positively or negatively (e.g., c-*myc*, *p53*, *bcl*-2, *Fas*/*Apo*-1, etc.) (Williams and Smith 1993). The tumor-suppressor *p53* gene is an essential component of the pathway leading from DNA damage to apoptosis (Lowe et al. 1993; Clarke et al. 1993), although it is still unknown how *p53* regulates two different functions: G1 growth arrest and apoptosis. Mutations in the *p53* gene is the most common genetic event found in human cancers, including gastric and rectal neoplasms (Hollstein et al. 1991).

Treatment strategies for patients with potentially curable gastric and rectal cancers remain primarily surgical. For years perioperative adjuvant chemotherapy and/or radiotherapy have been shown to improve the postoperative disease-free survival rate in these patients; the overall response rate, however, is less than ideal. Adjuvant therapy is still considered to be of little benefit to patients with gastric and rectal carcinomas compared to hematopoietic and pediatric malignancies that rarely involve *p53* mutations. Inactivation of the *p53* gene has been shown to contribute to enhanced cellular resistance to DNA-damaging treatment in vitro and in vivo (Lowe et al. 1993, 1994). These observations suggest that chemosensitivity and radiosensitivity in gastric and rectal cancers may be negatively modulated by frequent mutations in the *p53* gene.

In the present study we demonstrate that gastric and rectal carcinomas expressing mutated *p53* contain fewer apoptotic cells than those that have wild-type *p53* after treatments with anticancer drugs or radiation, suggesting that tumors with mutated *p53* are more resistant to preop-

erative chemotherapy and radiotherapy. The results support the possible clinical application of adenovirus-mediated transfer of the wild-type *p53* gene combined with DNA-damaging drugs for human cancer therapy that we previously reported (Fujiwara et al. 1994).

Materials and methods

Patients and tumor samples

Tumor samples were obtained from 24 patients with gastric or colorectal cancer who had undergone surgical resection at the Okayama University Hospital and the National Fukuyama Hospital. Three patients with gastric cancer were treated with systemic chemotherapy that consisted of intravenous doxorubicin (20 mg/m^2) on day 1, etoposide (100 mg/m^2) on days 4, 5, and 6, and cisplatin (40 mg/m^2) on days 2 and 8. Five patients with rectal cancer were treated with regional 30-Gy radiotherapy. Surgery was performed 2 weeks after preoperative treatments. The remaining 16 patients were surgically treated without any preoperative therapies. All tumor samples were fixed in 10% buffered formalin and embedded in paraffin. Paraffin blocks were then sectioned at 4 μm, the first section being routinely stained with hematoxylin and eosin for histological diagnosis and additional sequential sections being left unstained for immunohistochemical reactions.

Immunohistochemistry

Immunohistochemical staining was performed by using the Vectastain Elite kit (Vector, Burlingame, Calif.). The slides were immersed in 10 mM citrate buffer (pH 6) and autoclaved for a total of 15 min at 120 °C. The sections were blocked with 1% bovine serum albumin for 30 min and incubated with the primary antibody overnight at 4 °C in a high-humidity chamber. After a rinsing, the slides were incubated with biotinylated goat anti-(mouse immunoglobulin G) for 1 h and then with avidin-biotin-peroxidase complex for 30 min. Peroxidase activity was detected using 3,3′-diaminobenzidine tetrahydrochloride solution containing 0.003% hydrogen peroxide. Slides were counterstained with methyl green. The primary antibodies used were anti-p53 antibody and anti-WAF1 antibody (Oncogene Science, Manhasset, N.Y.). Negative control slides were processed with mouse preimmune immunoglobulin.

In situ detection of DNA fragmentation

Terminal deoxynucleotidyltransferase-mediated biotin-dUTP nick-end labeling (TUNEL) was performed according to a procedure reported previously (Gavrieli et al. 1992). Tissue sections were treated with 1 mg/ml proteinase K, immersed in terminal deoxynucleotidyltransferase buffer (30 mM TRIS/HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride), and then incubated with biotinylated dUTP (Boehringer Mannheim, Indianapolis, Ind.) and terminal deoxynucleotidyltransferase at 37 °C for 45 min. The slides were covered with 2% bovine serum albumin for 10 min and then incubated with avidin-biotin-peroxidase complex (Vector) for 30 min. The colorimetric detection was performed using diaminobenzidine. The frequency of apoptosis was quantified by determining the percentage of TUNEL-positive cells within a field at a magnification of ×400. For each slide, a total of five fields of nonnecrotic areas of the carcinoma were chosen randomly, and the numbers were averaged to obtain the apoptotic index.

Statistics

Statistical analysis was performed using Student's *t*-test for differences between groups. Statistical significance was defined as $P < 0.05$.

Table 1 Clinical and pathological characteristics of the patients. *PD* poorly differentiated adenocarcinoma; *MD* moderately differentiated adenocarcinoma; *WD* well-differentiated adenocarcinoma. *No treatment* no preoperative therapy; *EAP* preoperative chemotherapy that consisted of intravenous doxorubicin (20 mg/m^2) on day 1, etoposide (100 mg/m^2) on days 4, 5, and 6, and cisplatin (40 mg/m^2) on days 2 and 8; *Radiation* preoperative radiotherapy with 30 Gy. *Negative* fewer than 20% positive nuclei; *positive* at least positive nuclei

Patient	Age/sex	Tumor	Histology	Treatment	p53
1	73/F	Gastric ca	PD	No treatment	Negative
2	55/M	Gastric ca	WD	No treatment	Negative
3	69/M	Gastric ca	WD	No treatment	Negative
4	53/M	Gastric ca	PD	No treatment	Negative
5	42/M	Gastric ca	PD	No treatment	Negative
6	72/M	Gastric ca	MD	No treatment	Positive
7	60/F	Gastric ca	PD	No treatment	Positive
8	63/M	Gastric ca	MD	No treatment	Positive
9	54/M	Gastric ca	PD	EAP (2 courses)	Negative
10	50/M	Gastric ca	PD	EAP (2 courses)	Negative
11	36/F	Gastric ca	WD	EAP (2 courses)	Positive
12	60/M	Colon ca	MD	No treatment	Negative
13	72/F	Colon ca	WD	No treatment	Negative
14	53/M	Rectal ca	MD	No treatment	Negative
15	72/F	Colon ca	MD	No treatment	Negative
16	75/M	Colon ca	MD	No treatment	Positive
17	67/F	Colon ca	MD	No treatment	Positive
18	69/F	Colon ca	WD	No treatment	Positive
19	68/F	Rectal ca	MD	No treatment	Positive
20	62/M	Rectal ca	WD	Radiation	Negative
21	68/M	Rectal ca	WD	Radiation	Negative
22	75/F	Rectal ca	WD	Radiation	Negative
23	64/M	Rectal ca	MD	Radiation	Positive
24	74/M	Rectal ca	WD	Radiation	Positive

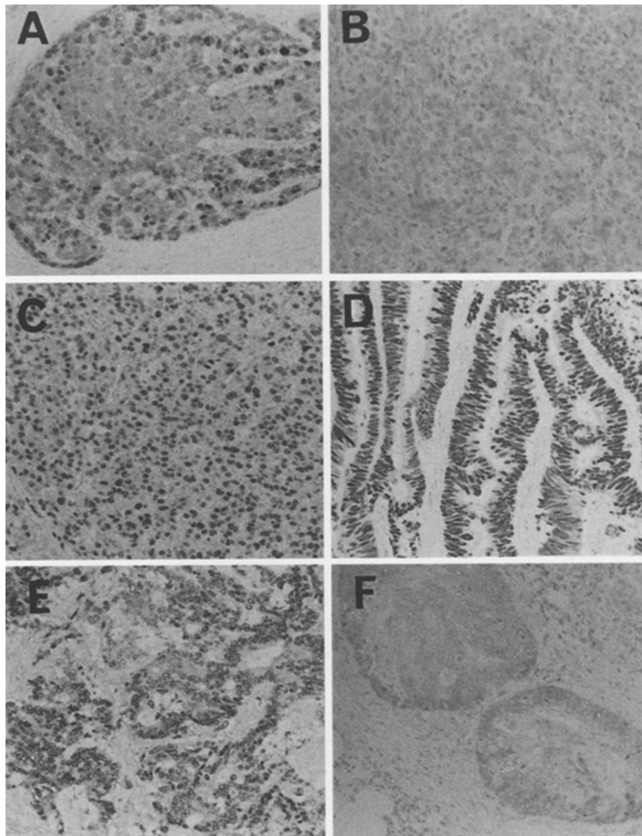
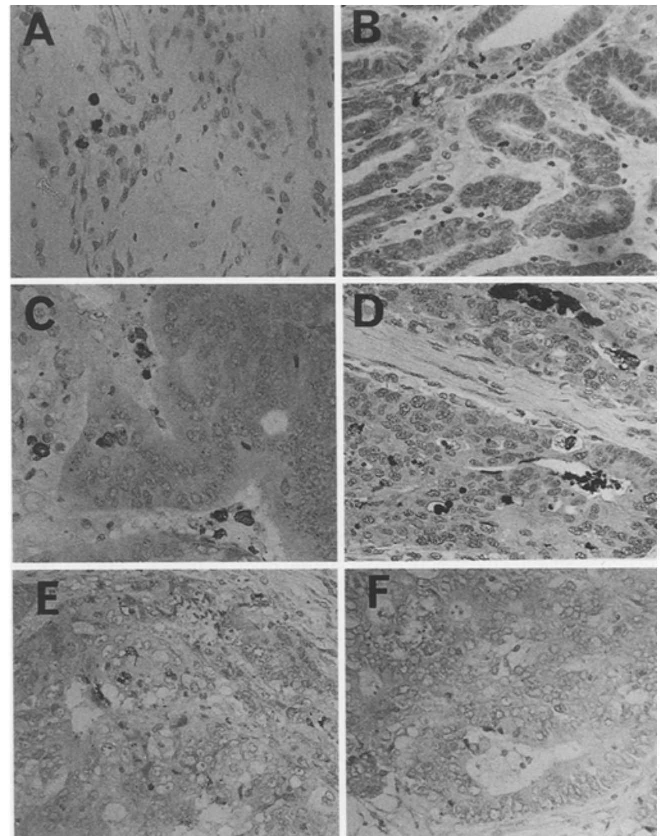


Fig. 1A–F Immunohistochemical staining for p53 protein. ×100. **A, C** p53-positive nuclear staining in gastric carcinomas (patients 6 and 11). **D, E** p53-positive staining in colorectal carcinomas (patients 18 and 24). **B, F** p53-negative gastric and rectal cancers (patients 9 and 20)

Results

Immunohistochemical staining for p53 oncoprotein

Immunohistochemical staining for p53 was performed on 24 paraffin-embedded gastric or colorectal cancer tissue specimens obtained from patients who underwent surgery. Patient and tumor characteristics are shown in Table 1. Nuclear staining of cells was considered positive for nuclear accumulation of p53 protein with a longer half-life, indicating the presence of *p53* mutation. Tumors were assigned to two p53-staining categories: negative (fewer than 20% positive nuclei) and positive (at least 20% positive nuclei). Positive staining for p53 was seen in 4 gastric cancers (36.4%) and 6 colorectal cancers (46.2%); thus, in total, 10 (41.7%) specimens were positive (Table 1). Three of the immunoreactive tumors were well-differentiated, 6 were moderately differentiated, and the remaining 1 was a poorly differentiated adenocarcinoma. Therefore, p53-positive staining was not associated with tumor types and histological grades. Positive neoplasms showed a diffuse immunostaining throughout the tumor (Fig. 1). No reactivity was observed in normal mucosa and stromal cells.



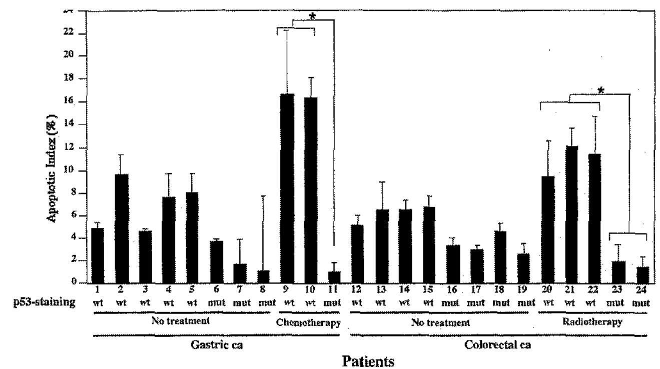


Fig. 2A–F Terminal deoxynucleotidyltransferase-mediated biotin-UTP nick-end labeling (TUNEL) staining for apoptotic cells. ×200. TUNEL-positive cells were visualized with a peroxidase-based color detection that produces a dark-brown staining. **A–C** Spontaneous apoptotic cells were observed in gastric and colorectal carcinomas (patients 5, 8, and 15). **D, E** Preoperative chemotherapy and radiotherapy induced many TUNEL-positive cells in p53-negative gastric and colorectal carcinomas respectively (patients 9 and 20). **F** No apoptotic cells were observed in p53-positive rectal tumors (patient 24). **G** The apoptotic index was determined for each tumor as described in Materials and methods. *Columns* mean; *bars* SD. $^{*}P<0.05$; Student's *t*-test

In situ detection of apoptosis in surgically resected tumor samples

Surgical materials were analyzed for apoptosis by the direct specific labeling of DNA fragmentation in nuclei. The micrographs of TUNEL assay are shown in Fig. 2A–F and the relationship between *p53* status and the apoptotic

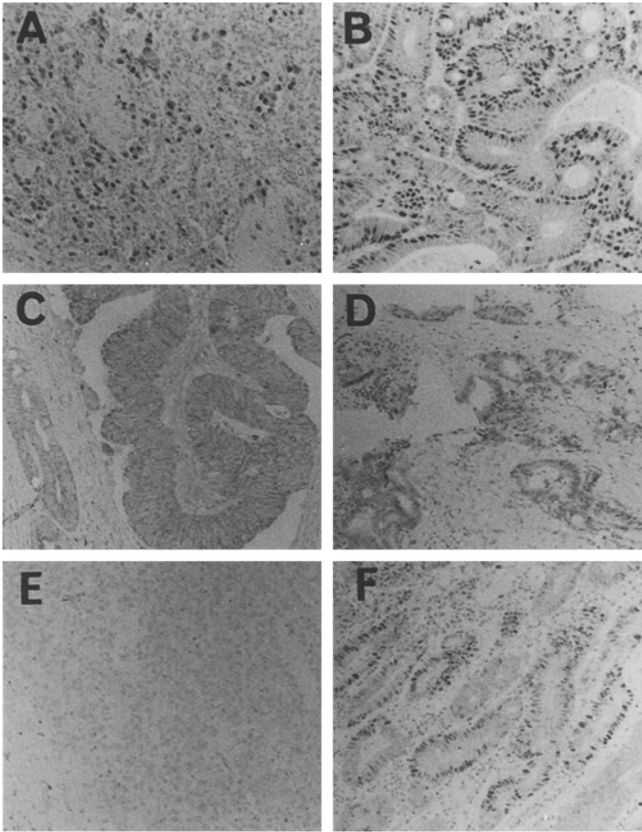


Fig. 3A–F Immunohistochemical staining for WAF1/CIP1 protein. ×100. **A, B** Spontaneous nuclear WAF1/CIP1 staining in gastric and colorectal tumors with wild-type *p53* (patients 3 and 15). **C** p53-positive tumors (patient 18) contained no WAF1/CIP1-positive cells. **D** WAF1/CIP1-positive nuclear staining in a p53-negative rectal tumor (patient 20). **E** The p53-positive tumor (patient 11) contained no WAF1/CIP1-positive cells even after preoperative chemotherapy. **F** WAF1/CIP1 protein was detectable in non-cancerous normal mucosa after preoperative systemic chemotherapy (patient 11)

index is illustrated in Fig. 2G. In gastric and colorectal cancers that received no preoperative treatment, the frequency of spontaneous apoptosis tended to be higher in tumors with no p53 immunoreactivity than in those overexpressing p53 protein (Fig. 2A–C): the mean apoptotic index was 6.7 ± 1.6% in p53-negative lesions compared to 2.9 ± 1.1% in p53-positive lesions (*P* not significant). Gastric neoplasms treated with chemotherapy and irradiated rectal carcinomas were also analyzed for apoptosis by TUNEL assay.

Two gastric cancer and 3 rectal cancer cases with no p53 immunoreactivity showed large clusters of TUNEL-positive cells after preoperative treatments; the number of positive cells was, however, very low in the p53-immunoreactive cases (1 gastric cancer and 2 rectal cancers) (Fig. 2D–F). A focal distribution of apoptotic cells was observed in locally irradiated rectal cancer, whereas in gastric cancer treated with systemic chemotherapy apoptotic cells were detected randomly. After chemo- or radiotherapy, the percentage of positive cells in tumors with wild-type *p53* ranged from 9.4% to 16.7% (13.2 ± 2.9%), whereas the percentage of apoptotic cells in mutant-*p53*-expressing tumors was 1.0%–1.9% (1.4 ± 0.4%) ($P < 0.05$). The TUNEL assay also demonstrated a higher apoptotic index in tumors that received chemotherapy or radiotherapy relative to those with no preoperative therapy (chemotherapy, 16.6 ± 0.2% versus 7.0 ± 2.0%, *P* not significant; radiotherapy, 11.0 ± 1.1% versus 6.26 ± 0.6%, $P < 0.01$) when tumors expressed wild-type *p53*. Most of the TUNEL-positive cells displayed characteristic apoptotic features with fragmented nuclei and chromatin condensation revealed by hematoxylin and eosin staining (data not shown). In histologically normal mucosa, TUNEL-positive cells are not observed.

Immunohistochemical staining for WAF1/CIP1 protein

A potential mechanism by which *p53* induces growth arrest has recently been proposed by demonstrating that *p53* regulates the expression of *WAF1/CIP1*, a potent inhibitor of cyclin-dependent kinases (Harper et al. 1993; El-Diery et al. 1993). Some cases of samples with no p53 oncoprotein expression, especially those containing more than 8% spontaneous apoptotic cells, showed a scattered pattern of WAF1/CIP1 staining; no nuclear WAF1/CIP1 staining was, however, immunohistochemically detected in mutant-*p53*-expressing tumors (Fig. 3). The *WAF1/CIP1* induction could also occur in the DNA-damage-response pathway, leading to apoptosis (El-Diery et al. 1994). The preoperative chemotherapy and radiotherapy induced WAF1/CIP1 protein expression in tumors with no p53 immunoreactivity, whereas tumors staining positively for p53 protein showed no WAF1/CIP1-positive cells although adjacent non-cancerous gastric or rectal mucosa contained some *WAF1/CIP1*-expressing cells (Fig. 3).

Discussion

Recent advances in our understanding of the molecular mechanism of apoptosis have provided the *p53* tumor-suppressor gene as a potential target for improved cancer therapies. Loss of *p53* activity through mutation or deletion could modulate the cytotoxic effects of antitumor agents such as chemotherapeutic drugs and ionizing radiation, leading the hypothesis that *p53* inactivations may provide a genetic basis for the therapeutic resistance to DNA-damaging cancer treatments (Kinzler and Vogelstein 1994). If the direct relation between *p53* mutations and therapeutic response can be identified in clinical cases, more benefit could be obtained from selecting tumors with wild-type *p53*, which therefore tend to respond well to DNA-damaging therapy. An association between the *p53* mutation and a poor prognosis has been demonstrated in several human cancers (Horio et al. 1993; Martin et al. 1992); however, to the best of our knowledge, the direct evidence that wild-type *p53* influences tumor responsiveness has not been proven in clinical samples. Because

induction of apoptosis may be a major factor determining the response of cancer to the therapy, we investigated the mode of cell death induced by preoperative chemotherapy or radiotherapy in gastric and rectal cancers.

The abnormalities of the *p53* gene were determined by an immunohistochemical assay. Previous studies have shown that the mutated form of p53 is much more stable than that of the wild type, partly because of an increase in the half-life of the protein (Harris and Hollstein 1993). Thus, immunohistochemical detection of p53 protein is well associated with specific *p53* mutations, although a heterogeneity of staining may often cause difficulties in further analyses of *p53*. In head and neck tumor and breast cancer, p53 expression has been reported to be very heterogeneous (Shin et al. 1994; Thompson et al. 1992); gastric and rectal neoplasms that we examined, however, showed a diffuse p53-positive phenotype throughout the tumor, indicating that most cells in the tumor lost the wild-type *p53* function (Fig. 1). The frequency of p53 protein accumulation in our series (41.7%) was somewhat lower than that previously reported for gastric and colorectal cancers (Yokozawa et al. 1992; Sinicrope et al. 1995). This may be due to the relatively small size of samples.

Cells undergoing apoptosis exhibit specific DNA degradation to oligonucleosomal sized fragments that can be detected by neutral gel electrophoresis. This method, however, involves homogenization of the entire cell population and may mask focal apoptotic changes in the tissue. Therefore, we used a direct labeling of DNA breaks in individual nuclei, a TUNEL procedure, to visualize areas of increased DNA fragmentation *in situ*. The tumor growth rate is defined by the balance of cell proliferation and cell death, and spontaneous apoptosis is a feature of human cancers. An inverse relationship between p53 immunoreactivity that indicates the *p53* mutations and the presence of apoptotic cells was observed in surgical specimens of human gastric and colorectal cancers. Moreover, our results indicate that tumors with p53 oncoprotein expression were significantly more likely to have a low apoptotic index than those with wild-type *p53* after preoperative anticancer therapy (Fig. 2), which is consistent with the predictions from experiments in murine model systems (Lowe et al. 1994). A clinical response of tumors to preoperative treatments was also influenced by the *p53* status. No evidence of reduction of the tumor size was noted in three p53-positive tumors, whereas a partial tumor regression following a marked decrease in carcinoembryonic antigen level was observed in 3 of 5 patients with p53-negative tumors (data not shown). These results suggest that *p53* gene mutations are associated with decreased chemo- and radiosensitivity, which may be related to an evasion of the wild-type-*p53*-mediated apoptosis, although an involvement of a *p53*-independent pathway in drug-induced apoptosis remains to be investigated.

The induction of the *WAF1/CIP1* gene, which that encodes a potent cyclin-dependent kinase inhibitor, has been demonstrated to occur in cells undergoing G1 growth arrest or apoptosis, and the degree of apoptosis induced by DNA-damaging agents correlated with the rate of WAF1/CIP1 protein accumulation in vitro (Fan et al. 1994). Thus, *WAF1/CIP1* is a critical downstream mediator in the *p53*-specific pathway. Our immunohistochemical staining showed that WAF1/CIP1 protein was detectable in the nucleus of all wild-type-*p53*-expressing, non-p53-immunoreactive tumors that contained scattered apoptotic cells after preoperative anticancer therapy (Fig. 3), which is the direct evidence that DNA-damaging therapy activates *WAF1/CIP1* expression in vivo through endogenous wild-type *p53* function. Interestingly, in tumors expressing mutant *p53*, carcinoma cells showed little WAF1/CIP1 staining, whereas epithelial cells in histologically normal mucosa adjacent to the tumoral tissue contained scattered WAF1/CIP1-positive cells after DNA-damaging treatments. TUNEL-positive, apoptotic cells, however, were not observed in these histologically normal areas (data not shown). These observations imply that DNA-damaging stimuli-induced in vivo *WAF1/CIP1* expression is not always associated with apoptosis, especially in the normal tissue, and may be responsible for the cell-cycle arrest. This is supported by the observation that some carcinomas with wild-type *p53* showed diffuse WAF1/CIP1 staining even without preoperative therapy, but contained a few apoptotic cells.

We previously proposed a novel strategy for human cancer gene therapy using replication-deficient wild-type *p53* adenovirus and a DNA-crosslinking agent, cisplatin (Fujiwara et al. 1994). Restoration of defective *p53* functions could restore the apoptotic response of cancer cells and increase their sensitivity to DNA-damaging agents. The present study provides a rationale for this therapy regimen. Gastrointestinal cancers (e.g., gastric and colorectal cancers) are still the main cause of cancer mortality. The therapeutic basis for surgery remains obvious; these tumors, however, vary in their sensitivity to other approaches such as chemotherapy and radiotherapy even when they are of the same histological type. Despite long-term trials of adjuvant therapy in advanced gastrointestinal cancer, optimal regimens leading to complete remissions have not been identified. A therapeutic goal of preoperative and/or postoperative adjuvant treatments is to direct tumors to apoptosis. Our results demonstrated that *p53* mutations negatively affected the tumor susceptibility to apoptosis. Thus, analysis of tumors for *p53* status may be of value in predicting the therapeutic response and selecting patients whose tumors harbor *p53* mutations for our gene therapy protocol.

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